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1) Mol Microbiol 1998 Apr;28(1):131-41
Aromatic ligand binding and intramolecular signalling of the phenol-responsive sigma54-dependent regulator DmpR.
O'Neill E, Ng LC, Sze CC, Shingler V.

2) J Bacteriol 1994 Dec;176(24):7550-7
An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of Pseudomonas sp. strain CF600 on para-substituted methylphenols.
Pavel H, Forsman M, Shingler V.

3) Biodegradation 1994 Dec;5(3-4):219-36
Genetics and biochemistry of phenol degradation by Pseudomonas sp. CF600.
Powlowski J, Shingler V.

4) J Bacteriol 1994 Aug;176(16):5052-8
Cross-regulation by XylR and DmpR activators of Pseudomonas putida suggests that transcriptional control of biodegradative operons evolves independently of catabolic genes.
Fernandez S, Shingler V, De Lorenzo V.

Thank you,
David Steadman

Molecular Microbiology (1998) 28(1), 131–141

Aromatic ligand binding and intramolecular signalling of the phenol-responsive σ^{54}-dependent regulator DmpR

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Summary

The *Pseudomonas*-derived σ^{54}-dependent regulator DmpR has an amino-terminal A-domain controlling the specificity of activation by aromatic effectors, a central C-domain mediating an ATPase activity essential for transcriptional activation and a carboxy-terminal D-domain involved in DNA binding. In the presence of aromatic effectors, the DmpR protein promotes transcription from the −24, −12 Po promoter controlling the expression of specialized (methyl)phenol catabolic enzymes. Previous analysis of DmpR has led to a model in which the A-domain acts as an interdomain repressor of DmpR's ATPase and transcriptional promoting property until specific aromatic effectors are bound. Here, the autonomous nature of the A-domain in exerting its biological functions has been dissected by expressing portions of DmpR as independent polypeptides. The A-domain of DmpR is shown to be both necessary and sufficient to bind phenol. Analysis of phenol binding suggests one binding site per monomer of DmpR, with a dissociation constant of 16 μM. The A-domain is also shown to have specific affinity for the C-domain and to repress the C-domain mediated ATPase activity *in vitro* autonomously. However, physical uncoupling of the A-domain from the remainder of the regulator results in a system that does not respond to aromatics by its normal derepression mechanism. The mechanistic implications of aromatic non-responsiveness of autonomously expressed A-domain, despite its demonstrated ability to bind phenol, are discussed.

Introduction

Members of the prokaryotic σ^{54}-dependent family of regulators resemble eukaryotic transcription factors in exerting their action from a distance and having a modular structure (Kustu *et al.*, 1991; Morett and Segovia, 1993; see Fig. 1). The distinct domains of this family of transcriptional activators mediate specific function(s) and exhibit varying degrees of homology (reviewed by Morett and Segovia, 1993). Typically, the amino-terminal signal reception A-domains are separated from the central activation C-domains by a short B-domain that serves as a flexible linker. The C-domains are variably distanced from the conserved carboxy-terminal D-domains that contain a helix–turn–helix DNA-binding motif. The highly conserved central C-domains of the regulators contain a nucleotide binding motif and mediate ATP binding and hydrolysis essential for transcriptional activation and open complex formation by σ^{54}-RNA polymerase (Austin and Dixon, 1992; Weiss *et al.*, 1991). In some cases, the C-domain has also been shown to mediate oligomerization required before ATPase activity and, thus, transcriptional activation (Porter *et al.*, 1993; 1995; Perez-Martin and de Lorenzo, 1996a), a process that is facilitated by binding to its cognate enhancer sequence (Mettke *et al.*, 1995; Perez-Martin and de Lorenzo, 1996a). The C-domain is also believed to encompass the region involved in direct interaction with σ^{54}-RNA polymerase and, thereby, couples ATP hydrolysis and transcriptional activation. The mechanism underlying this coupling is not fully understood but may involve a process by which ATP binding and hydrolysis mediate conformational changes that allow the regulator to interact successfully with σ^{54} and, thereby, relieve the repressive effect of σ^{54} on the ability of core RNA polymerase to form open transcriptional complexes (Wang *et al.*, 1995; Perez-Martin and de Lorenzo, 1996a; Wang and Gralla, 1996).

The A-domain signal reception module defines different subgroups of σ^{54}-dependent regulators, which reflect distinct mechanisms by which the activity of this family of regulators is controlled (reviewed by Shingler, 1996). Stimulation of the regulatory activity of many members of the family, including the two archetypal members NtrC and DctD, is achieved by phosphorylation of a conserved Asp residue of the A-domain in response to signals detected by specific sensory histidine kinases. The activities of another subgroup of the family, including NifA and some NifA-like proteins, are controlled by signal responsive protein–protein interactions. DmpR, however, belongs to the rapidly growing mechanistic subgroup that directly senses

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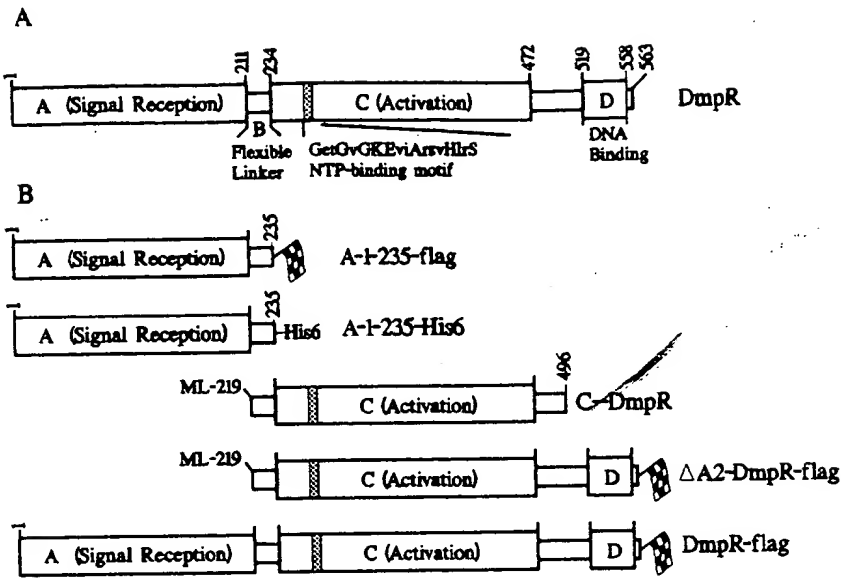

Fig. 1. A. Schematic representation of the functional domains of DmpR discussed in the text. The extent of the NTP binding site consensus sequence (given in upper case letters) is indicated by a shaded box.
B. Schematic illustration of key DmpR derivatives used to analyse the A-/C-domain interaction of DmpR in this study.

and responds to small effector molecules (Shingler and Moore, 1994; Shingler and Pavel, 1995) and includes the aromatic responsive regulators XylR (Abril *et al.*, 1989; Delgado and Ramos, 1994), PhhR (Ng *et al.*, 1995), TbuT (Byrne and Olsen, 1996), MopR (Schirmer *et al.*, 1997), the formate-responsive FhlA (Hopper and Böck, 1995) and the ornithine/citrulline-responsive RocR (Gardan *et al.*, 1997).

The physiological role of DmpR is to control the expression of specialized (methyl)phenol catabolic enzymes encoded by the 15 gene *dmp* operon (Shingler *et al.*, 1992; 1993). The transcriptional promoting activity of DmpR is activated in the presence of the *dmp* pathway substrates and some, but not all, structural analogues (Shingler and Moore, 1994). Chimeric DmpR/XylR proteins (Shingler and Moore, 1994) and isolation of effector specificity mutants (Pavel *et al.*, 1994; Shingler and Pavel, 1995) identified the A-domain as incorporating all the determinants required to mediate the specificity of activation of DmpR by its aromatic effectors. Interaction of DmpR with its activating ligand results in the expression of its otherwise repressed ATPase activity (Shingler and Pavel, 1995). Moreover, effector-mediated derepression of the C-domain ATPase and transcriptional promoting activity can be mimicked by deletion of the A-domain (Shingler and Pavel, 1995). These observations led to a model in which the A-domain serves to mask the C-domain-mediated ATPase activity of DmpR in the absence of effectors.

Within DmpR, single amino acid changes in the A-domain, the central activation C-domain and the short flexible B-linker domain result in proteins with varying degrees of activity in the absence of effector (Shingler and Pavel, 1995). These semi-constitutive mutations, therefore, partially mimic the activated state of the protein. The locations of the mutations suggested that the A-domain may mediate its repressive function by A/C interdomain interactions that are tethered via the normally flexible B-domain. Genetic analysis, involving the isolation of second-site mutations in the A-domain that suppressed the phenotype of a C-domain-located semi-constitutive mutant and vice versa (Ng *et al.*, 1996), expanded the model to include specific residue–residue interactions between the two domains in the absence of effector.

In this report, we extend the model of ligand binding and intramolecular signalling of DmpR by demonstrating (i) specific interaction between the A- and C-domains of DmpR that is independent of the physical continuity of the two domains; (ii) that physically uncoupled A-domain can specifically repress the ATPase activity of DmpR derivatives *in vitro*; (iii) that the A-domain contains the phenol binding site; and (iv) that the repressive property of uncoupled A-domain is not alleviated by the addition of aromatic effectors. These findings all support a model of DmpR in which the amino-terminal A-domain functions autonomously in phenol binding and interdomain repression, but its derepression response requires physical continuity with the rest of the regulator.

Results

Autonomous repression by the A-domain of DmpR on the ATPase activity of the C-domain in vitro

As the first step in determining if the amino-terminal A-domain of DmpR contains an autonomous repressive activity, we generated the T7 promoter-driven plasmid pVI510, which expresses A-1-235-His6 encompassing the amino-terminal 235 amino acid residues of DmpR fused, with an intervening Ser residue, to six consecutive

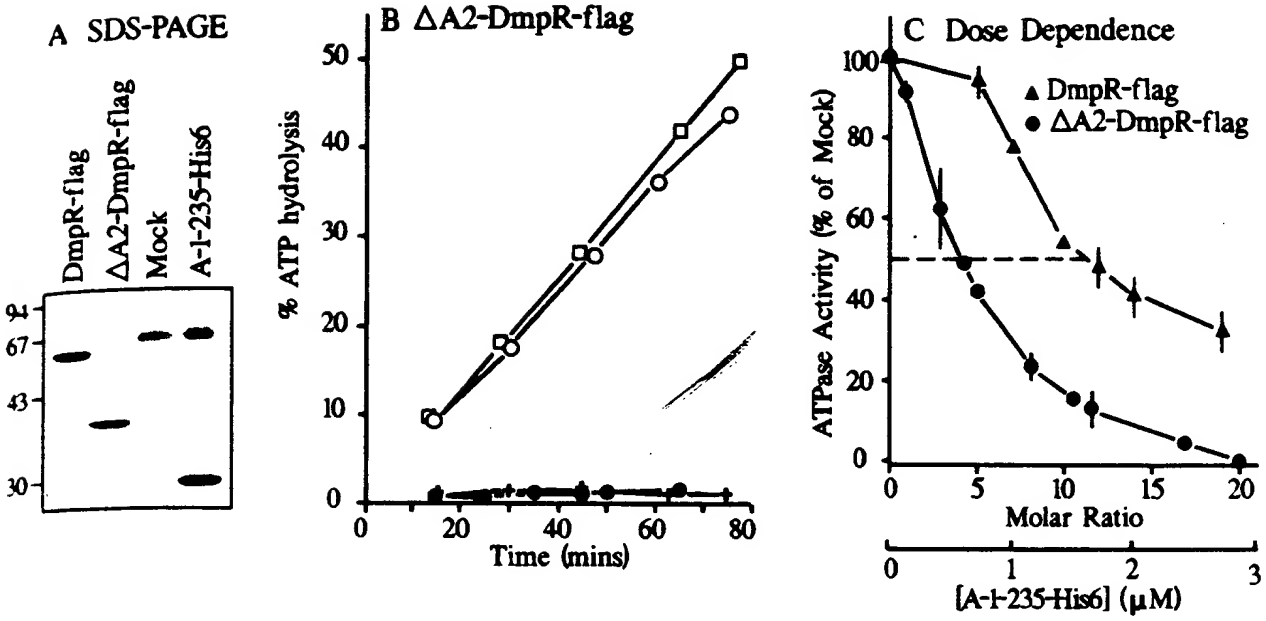

Fig. 2. Regulation of the ATPase activity of ΔA2-DmpR-flag and DmpR-flag by A-1-235-His6.
A. Coomassie blue-stained 10–20% SDS–PAGE of the protein preparations isolated as described in *Experimental procedures*; DmpR-flag, 64.4 kDa (3.0 μg released from 5 μl of beads); ΔA2-DmpR-flag, 39.4 kDa (2.0 μg released from 5 μl of beads); A-1-235-His6, 28 kDa (3.0 μg, 12.5 μl of BSA buffer). For the mock preparation, an equivalent volume to that of A-1-235-His6 was loaded. The band observed at 67 kDa in the mock and A-1-235-His6 preparations is the acetylated BSA added during purification to prevent precipitation of A-1-235-His6 upon storage (see *Experimental procedures*).
B. ATP hydrolysis mediated by 1 μl (0.4 μg) of bead-bound ΔA2-DmpR-flag in the presence of 24 μl of mock (□); 24 μl (3.3 μM) of A-1-235-His6 (●); 24 μl (3.3 μM) of A-1-235-His6 and 1 mM (300-fold molar excess) 2-methylphenol (+); or 24 μl of A-1-235-His6 after 10 min boiling (○). Assays were performed as described in *Experimental procedures*, and the data are the average of duplicate experiments.
C. Dose–response of the inhibitory property of A-1-235-His6 on ΔA2-DmpR-flag and DmpR-flag. The kinetics of ATPase reactions were analysed as in (B) with 1 μl of bead-bound ΔA2-DmpR-flag or DmpR-flag and 24 μl of A-1-235-His6 preparation diluted appropriately with the mock solution. ATP hydrolysis is expressed as a percentage of that observed with ΔA2-DmpR-flag or DmpR in the presence of mock preparation only. Data are the average from two independent experiments.

His residues (see Fig. 1). Cells expressing A-1-235-His6 were used for purification of the protein as described in *Experimental procedures*, resulting in a highly enriched preparation, depicted in Fig. 2A.

To assess the ability of A-1-235-His6 to mediate its regulatory role, we made use of a previously optimized *in vitro* ATPase assay based on bead-bound affinity-purified ΔA2-DmpR-flag, which is constitutively active because of deletion of the A-domain (Shingler and Pavel, 1995). These two proteins, A-1-235-His6 and ΔA2-DmpR-flag, include all the residues of DmpR separated into two polypeptides (see Fig. 1). As shown in Fig. 2B, A-1-235-His6 was found to repress the ATPase activity of ΔA2-DmpR-flag completely. The A-1-235-His6 polypeptide aggregates easily, which necessitates the use of detergents and BSA to keep the protein in solution during purification (see *Experimental procedures*). Therefore, to control for the effects of these components and the low level of protein contaminants in the A-1-235-His6 preparation, a mock preparation from cells harbouring a vector control was prepared. The ATPase activity of ΔA2-DmpR-flag in the presence of this mock preparation (Fig. 2B) did not significantly differ from that previously found for ΔA2-DmpR alone (data not shown; Shingler and Pavel, 1995). Moreover, boiling of the A-1-235-His6 preparation completely removed the repressive activity (Fig. 2B). As an additional specificity control, we generated a constitutively ATPase-active derivative of σ^{54}-dependent NifA from *Klebsiella pneumoniae* (Buchanan-Wollaston *et al.*, 1981) (ΔA-NifA-flag; see *Experimental procedures*). Neither A-1-235-His6 nor the mock preparation were found to have any effect on the ATPase activity of ΔA-NifA-flag (data not shown). Therefore, we conclude that the inhibition of the ATPase activity of ΔA2-DmpR-flag by the A-1-235-His6 preparation is mediated specifically by the repressive activity of the A-1-235-His6 polypeptide and is not caused by contaminants or buffer composition.

The results depicted in Fig. 2B demonstrate that the amino-terminal region of DmpR is an autonomous domain that can exert its regulatory repressive function when physically uncoupled from the remainder of the protein. To test if this system is also autonomous with respect to response to the addition of aromatic effectors of DmpR, 1 mM of the most efficient effector of DmpR, 2-methylphenol, was added in the two-component ATPase repression assay. This concentration of effector was chosen, as it elicits

maximal *in vitro* activation of DmpR-flag, while having no detrimental effects on the constitutive ATPase activity of ΔA2-DmpR-flag (Shingler and Pavel, 1995) or ΔA-NifA-flag (data not shown). As shown in Fig. 2B, using 3.3 μM A-1-235-His6, which mediates 100% repression, the presence of effector was found to have no alleviating effect. Preincubation of A-1-235-His6 and/or ΔA2-DmpR-flag with effector did not influence the result (data not shown). As the effector is in 300- to 2000-fold excess over A-1-235-His6 in these experiments, insufficient effector is unlikely to be responsible for the effector non-responsive property.

The finding that DmpR effectors failed to alleviate the repression mediated by A-1-235-His6 on ΔA2-DmpR-flag leads to the prediction that this polypeptide might also be able to repress the activity of intact DmpR, despite its contiguous native A-domain and the requirement for effector to derepress its ATPase activity. To test this prediction, the dose–response to different concentrations of A-1-235-His6 on the effector-derepressed ATPase activity of DmpR-flag was compared with that on constitutively active ΔA2-DmpR-flag. As shown in Fig. 2C, A-1-235-His6 is capable of repressing the ATPase activity of both proteins in a dose-dependent manner. However, approximately threefold more A-1-235-His6 is required to achieve 50% repression of DmpR-flag than was observed for ΔA2-DmpR-flag. In addition, complete repression of DmpR-flag could not be achieved. The differences between the A-1-235-His6 dose–responses of DmpR-flag and ΔA2-DmpR-flag are most probably attributable to the presence of DmpR-flag's native A-domain, which may hinder accessibility and will compete with exogenous A-1-235-His6 for its interactive surface on DmpR.

It should be noted that the addition of the mock preparation did not alter the specific activity of effector-derepressed DmpR-flag (data not shown; Shingler and Pavel, 1995), i.e. the native A-domain of DmpR responds normally under the buffer conditions used. This result rules out the trivial possibility that the detergents and/or BSA present in the A-1-235-His6 (and mock) preparations are responsible for the effector non-responsive property of A-1-235-His6.

Interaction between non-contiguous A- and C-domains of DmpR can be detected in vivo

The results outlined above demonstrate that the 235 amino acids of the A-domain are sufficient for specific interaction with the C-domain of DmpR to repress its ATPase activity *in vitro*. This predicts that uncoupled A-domain has direct affinity for the C-domain. To test this idea, we analysed the ability of physically uncoupled A- and C-domains to form complexes *in vivo* that can be detected by co-immunoprecipitation. To this end, we used compatible plasmids that express A-1-235-flag (pVI521), DmpR (pVI531) or just the C-domain of DmpR (C-DmpR, pVI532) in *P. putida*. Crude extracts were prepared from cultures expressing each of the proteins alone or co-expressing A-1-235-flag with either DmpR or C-DmpR. Immunoprecipitation was performed on the basis of the flag epitope of A-1-235-flag in the presence or in the absence of effector. Proteins of the crude extract (Fig. 3, left) and protein complexes isolated by immunoprecipitation (Fig. 3, right) were separated by SDS–PAGE and subjected to Western analysis using antibodies directed either towards the C-/D-domains of DmpR, the flag epitope tag or, as a specificity control, towards TrpE. The results summarized in Fig. 3 demonstrate that both DmpR and C-DmpR are specifically co-immunoprecipitated with A-1-235-flag. The inclusion of C-DmpR in this analysis ensures that the physical association observed between the two players is attributable to the A-/C-domain interaction. However, it is possible that additional contacts between the A-domain and other parts of the regulator also contribute to the co-precipitation of DmpR. Nevertheless, in neither case did the addition of effector abolish the physical association. Thus, like the

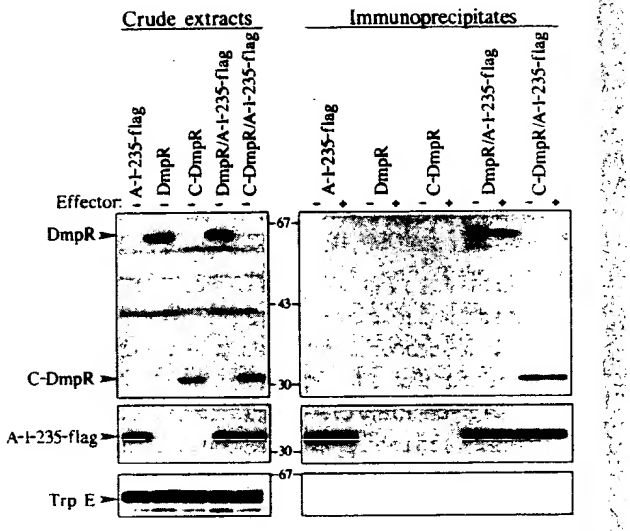

Fig. 3. Co-immunoprecipitation of DmpR and C-DmpR by A-1-235-flag in the presence (+) and absence (−) of 1 mM 2-methylphenol. Autoradiograph of Western blot analysis of 11% SDS–PAGE separated preparations of crude extracts (30 μg, left), prepared from strains expressing the indicated proteins and proteins retrieved using anti-flag M2-affinity beads (5 μl of bead-bound protein, right), as described in *Experimental procedures*. In the experiment shown, 1 mM 2-methylphenol was added to a sample of crude extract and was present through all the following steps. Similar data were obtained in experiments in which the initial culture was also grown in the presence of effector. Arrows indicate the locations of DmpR and C-DmpR polypeptides, revealed using anti-DmpR sera generated against a denatured polypeptide with both the C- and D-domains of DmpR (top), A-1-235-flag visualized using the anti-flag M2 monoclonal antibody (middle) and TrpE used as a control, visualized using an anti-TrpE sera (bottom). Note that some of the epitopes present in DmpR are missing in C-DmpR. Standards are given in kDa.

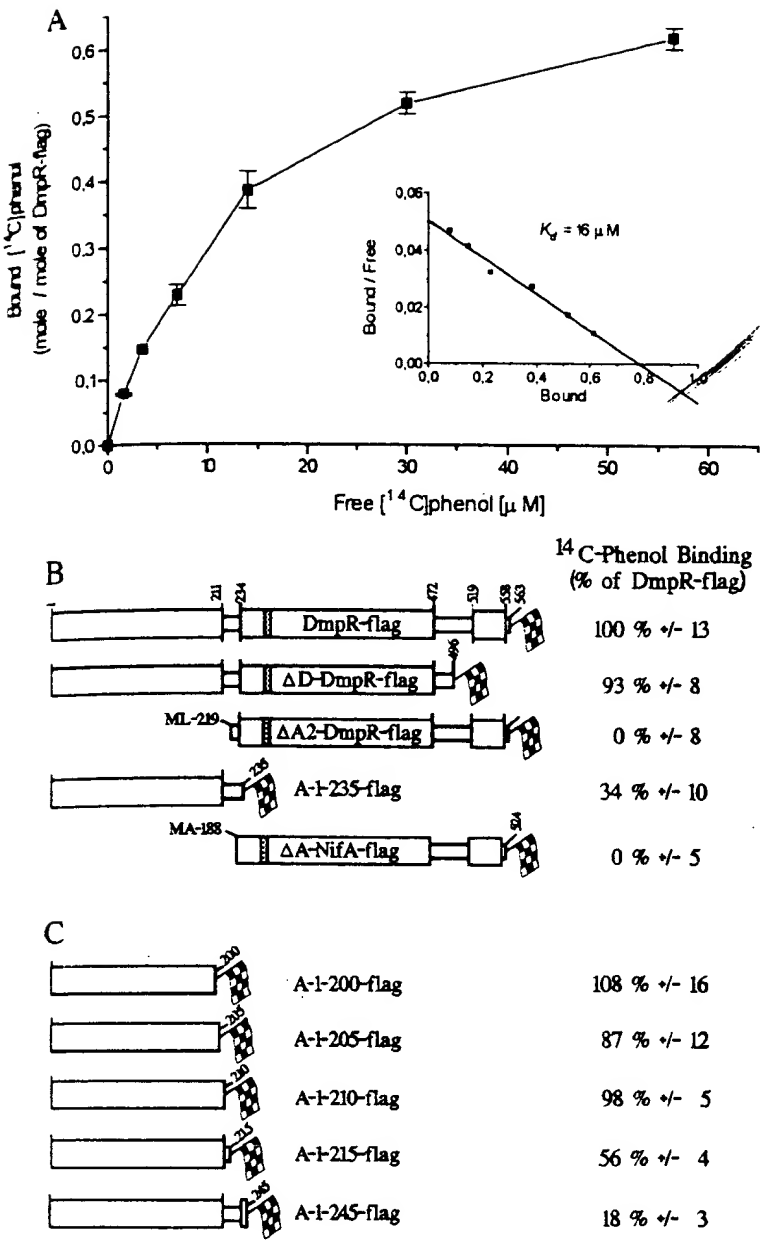

Fig. 4. [^{14}C]-phenol binding.
A. Binding curve for [^{14}C]-phenol by DmpR-flag determined as described in *Experimental procedures*. Data are the average of triplicate determinations in two independent experiments. Insert shows Scatchard analysis used to derive the dissociation constant (K_d = 16 μM) of DmpR-flag for phenol.
B. [^{14}C]-phenol binding by derivatives used to demonstrate that the A-domain is necessary for phenol binding.
C. [^{14}C]-phenol binding by derivatives used to demonstrate that the A-domain is sufficient for phenol binding. Data are the average of duplicate determinations.

repression mediated by the A-domain when present as a separate polypeptide in the ATPase repression assay, the physical association observed *in vivo* using a co-immunoprecipitation assay is also effector non-responsive.

The A-domain of DmpR is both necessary and sufficient to bind phenol

The effector non-responsive property of A-1-235-His6 suggests that either the A-domain is insufficient to interact with the aromatic effector or, alternatively, that physical uncoupling of the A-domain renders it incapable of transducing the intramolecular signal to derepress the system. As a first step in testing the former of these two possibilities, we set up a system using [^{14}C]-phenol and bead-bound DmpR-flag to analyse phenol binding (see *Experimental procedures*). The data shown in Fig. 4A demonstrate specific and saturatable binding of phenol by DmpR. Evaluation of the binding data by Scatchard analysis suggests (i) a single class of binding sites; (ii) that phenol is bound with a dissociation constant of 16 μM; and iii) that 0.8 mol of phenol are bound per mol of protein, which is compatible with one binding site per monomer of DmpR.

To determine if the first 235 residues of DmpR encompass the phenol binding site, we used the [^{14}C]-phenol binding assay and the series of DmpR-flag-tagged derivatives

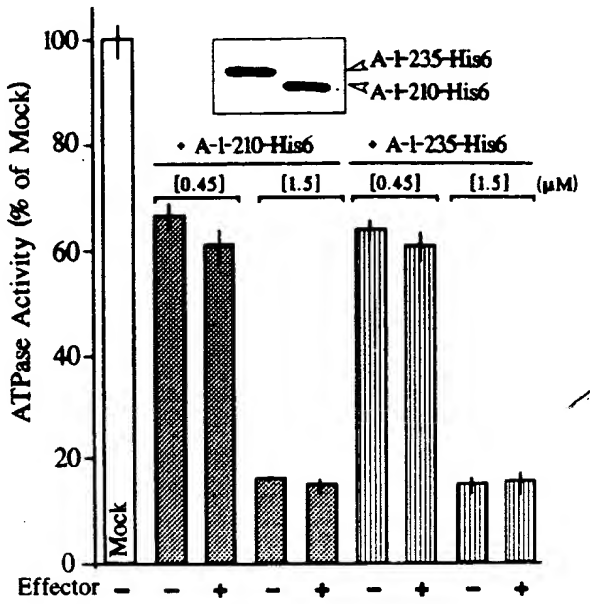

Fig. 5. Effector non-responsiveness of repression mediated by A-1-210-His6 and A1-235-His6 on the ATPase activity of ΔA2-DmpR-flag. The kinetics of ATPase activity were determined as described in Fig. 2. The repression mediated by two different concentrations (0.45 and 1.5 μM, as indicated) of each of the A-domain-His6 polypeptides in the presence (+) and in the absence (–) of 1 mM 2-methylphenol is expressed as a percentage of the rate of ATP hydrolysis observed with ΔA2-DmpR-flag in the presence of a mock preparation. Data are the average of two experiments. Insert shows a Coomassie blue stain of 50 pmol of the A-1-235-His6 and A-1-210-His6 preparations separated by 10–20% SDS–PAGE.

depicted in Fig. 4B, with ΔA-NifA-flag as a negative control. As summarized in Fig. 4B, deletion of the DNA binding D-domain of DmpR had no significant effect on phenol binding, while deletion of the A-domain resulted in a protein completely unable to bind phenol. A-1-235-flag, spanning the residues of DmpR present in A-1-235-His6 used in the assays described in preceding sections, was found to be able to bind phenol, but only to 34% of the level achieved by DmpR-flag.

To determine which residues were required to give wild-type levels of phenol binding, we generated the series of flag-tagged polypeptides depicted in Fig. 4C. Residues 1–235 of DmpR, which mediated 34% of phenol binding, encompass the short flexible B-linker in addition to the A-domain of DmpR (see Fig. 4B). An additional 10 residues in A-1-245-flag were found to decrease phenol binding down to 18%. This suggested that the flexible linker region present in these proteins, which is fused to the C-terminal flag sequence, may interfere with phenol binding in some way. To test this idea, we generated four constructs that express flag-tagged derivatives encompassing the first 200–215 amino acid residues of DmpR. Binding of phenol to the same extent as the intact regulator was found with the three derivatives that did not have any of the residues of the B-linker (A-1-210-flag, A-1-205-flag and A-1-200-flag). Hence, the first 200 residues of DmpR contain the phenol binding site of DmpR.

The finding that A-1-210-flag, which encompasses the A-domain alone, bound wild-type levels of phenol, prompted us to compare the aromatic-responsiveness of this A-domain derivative expressed as A-1-210-His6 with those of A-1-235-His6 in the two-component ATPase repression assay. As shown in Fig. 5, purified A-1-210-His6 was found to repress the ATPase activity of ΔA2-DmpR-flag with the same dose-dependency as A-1-235-His6. Similarly, the repression mediated by A-1-210-His6 was not responsive to the presence of aromatic effectors (Fig. 5). Thus, we conclude that the A-domain of DmpR is capable of autonomous interdomain repression and phenol binding, but physical uncoupling of the A-domain renders it unable to couple phenol binding to derepression.

Discussion

The regulation of constitutively expressed transcriptional activators provides a mechanism for an immediate response to environmental and physiological signals. Repression is a common mechanism by which the intrinsic activity of different families of prokaryotic transcriptional activators are kept silent until the activities of the genes they control are required. For example, the normal activating signal is not required when various portions of AraC (Menon and Lee, 1990), FruJ (Kahn and Ditta, 1991), LuxR (Choi and Greenberg, 1991) or Spo0A (Ireton *et al.*, 1993) are deleted. Similarly, the DNA binding properties of σ^{70} and related sigma factors are also under repression control (Dombroski *et al.*, 1993). DmpR (Shingler and Pavel, 1995) belongs to a mechanistic subgroup of σ^{54}-dependent regulators which includes XylR (Delgado *et al.*, 1995; Fernández *et al.*, 1995), MopR (Schirmer *et al.*, 1997), DctD (Gu *et al.*, 1994), FhlA (Korsa and Böck, 1997) and RocR (Gardan *et al.*, 1997), in which deletion and/or mutation analysis has implicated intramolecular repression as a common control mechanism (for review, see Shingler, 1996).

In this work, we have investigated the mechanism by which the transcriptional promoting ability of phenol-responsive DmpR and its intrinsic ATPase activity are kept in check by the amino-terminal of the regulator. As outlined in the *Introduction*, the key control event is direct interaction of aromatic effectors with DmpR, which leads to the expression of its otherwise repressed C-domain-mediated ATPase activity (Shingler and Pavel, 1995). Hence, effector binding and derepression are intimately related. Previous work has implicated the A-domain of DmpR and that of the closely related regulator XylR in both these processes. First, the A-domain has been shown to mediate the specific aromatic effector profiles of DmpR and XylR (Delgado and Ramos, 1994; Shingler

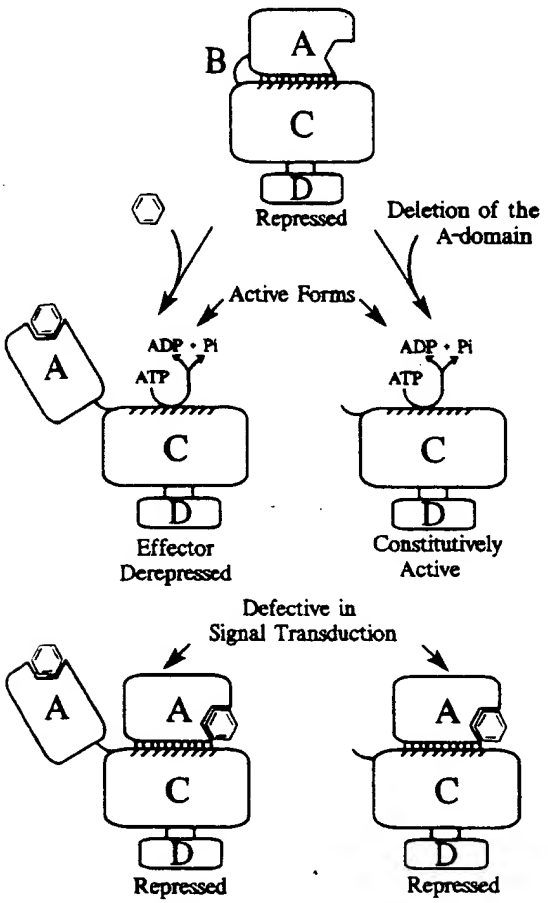

Fig. 6. Model for repressive control of the activity of DmpR by its A-domain in the native regulator (left) or physically uncoupled system (right). A, B, C and D represent the functional domains as described in the text. Vertical bars represent residue–residue interactions between the A- and C-domains, and hatched regions represent the interactive surface on the C-domain that is masked by the A-domain, resulting in repression of its ATPase activity and transcriptional promoting property.

and Moore, 1994; Shingler and Pavel, 1995). Secondly, deletion of the A-domain of DmpR and XylR results in derivatives that no longer require effectors and have full constitutive ATPase and transcriptional promoting activity (Fernández *et al.*, 1995; Shingler and Pavel, 1995; Perez-Martin and de Lorenzo, 1996b). Together, these findings indicated a repressive function for the A-domain on the activity of the C-domain in the absence of effectors, rather than an activating role in the presence of effectors (see Fig. 6). This mechanism is also supported by the effector-independent phenotype of DmpR and XylR derivatives harbouring mutations in either the A- or the C-domains (Delgado *et al.*, 1995; Shingler and Pavel, 1995) or in the short linker B-domain that separates them (Fernández *et al.*, 1995; Shingler and Pavel, 1995). Previous evidence suggesting that A/C interdomain interactions are responsible for repression has been obtained for DmpR by the isolation of reciprocal second-site mutations in the alternate domains (Ng *et al.*, 1996) and for XylR by *in vivo* inhibition of a constitutively active derivative by overexpression of its cognate A-domain (Perez-Martin and de Lorenzo, 1995a). Here, an assay that uses two affinity-purified polypeptides, which encompass all the residues of DmpR but physically separate the A-domain from the rest of the regulator, was used to show that the A-domain of DmpR is an autonomous interdomain repressor of DmpR's ATPase activity *in vitro* (Figs 2 and 5). Furthermore, using *in vivo* co-expression in the native host *P. putida*, the strength of the physical association between the A- and C-domains was found to be sufficient to allow co-immunoprecipitation (Fig. 3). Hence, both *in vitro* and *in vivo* data reveal a significant affinity between the A- and C-domains of DmpR.

Relief of A-domain repression by binding of the aromatic effector is the flip side of the mechanism that controls the activity of DmpR. The repression mediated by micromolar concentrations of uncoupled A-domain *in vitro* is non-responsive to the addition of a millimolar concentration of effector (Figs 2B and 5). Nor was the presence of a large excess of effector able to break the physical association observed *in vivo* between the A- and C-domains of DmpR by co-immunoprecipitation (Fig. 3). In agreement with these data, *in vivo* inhibition of derivatives of the cognate regulator by overexpression of the A-domains of XylR (Perez-Martin and de Lorenzo, 1995a) and MopR (Schirmer *et al.*, 1997) *in trans* have recently been shown to be unaffected by the addition of effectors. Hence, for DmpR and, in all probability, for XylR and MopR, effector non-responsiveness of the physically uncoupled A-domain is an intrinsic property. These data lead to the conclusion that either the A-domain is insufficient to bind effector or it is incapable of intramolecular signalling in the absence of physical continuity with the rest of the protein.

In order to resolve the two alternative models, we mapped the region required for phenol binding by DmpR. The identification of the A-domain as encompassing all the necessary determinants to mediate the specificity of DmpR derepression in response to aromatics with different substituents (Shingler and Moore, 1994) implicated this domain in aromatic effector binding. However, in no case had the effector binding property of any ligand-responsive σ^{54}-dependent regulator been assessed directly. Thus, it was conceivable that the A-domain binds, or stabilized binding, via substituents of the aromatic ring and, thereby, determining specificity, while other parts of the regulator are also involved in the binding of the core aromatic ring. However, here we show that the A-domain is both necessary and sufficient to bind phenol, and the data suggest that it contains a single binding site for its aromatic ligand (Fig. 4). Hence, the data confirm that the A-domain of DmpR constitutes a discrete domain, as it could be expressed and purified as a protein with two distinct

biological activities, namely phenol binding and inter-domain repression.

The autonomous ability of the A-domain to bind phenol in turn leads to the conclusion that aromatic non-responsiveness of the physically uncoupled A-domain is caused by lack of linkage to the C-domain, with consequent failure in signal transduction, as depicted in Fig. 6. Analysis of phenol binding by carboxy-terminal flag-tagged derivatives of DmpR that contain the A-domain and different portions of the B-linker led to the interesting observation that possession of B-linker residues interfered with phenol binding (Fig. 4). As the proteins were bound to beads via the flag tag epitope, this result suggests that the linker region may be involved in modulating the accessibility of the A-domain phenol binding site. Two lines of evidence had previously implicated the short flexible B-linker as a mechanistic component of intramolecular signalling. First, the partially constitutive, although still effector-responsive, phenotype of DmpR and XylR derivatives harbouring disruptive proline substitutions or insertions within the linker region suggests the importance of flexibility within this region in allowing the correct positioning of the A-domain on its C-domain interactive surface for repression (Fernández *et al.*, 1995; Shingler and Pavel, 1995). Secondly, deletion analysis of XylR and MopR, which respond to aromatic effectors, and the phosphorylation-responsive DctD have mapped the carboxy-terminal portions of their A-domains as the regions involved in keeping the respective regulators silent (Gu *et al.*, 1994; Perez-Martin and de Lorenzo, 1995b; Schirmer *et al.*, 1997). The location of these inhibitory regions adjacent to the short flexible B-linker may suggest that the linker acts as a pivot point at which the interactions that mediate repression are broken upon effector binding. Alternatively, by analogy with NtrC (Fiedler and Weiss, 1995), promotion or inhibition of putative A-domain dimer formation in the absence of physical continuity with the rest of the regulator may underlie the inability of physically uncoupled A-domain to transduce the aromatic effector binding signal. Elucidation of the mechanism of phenol-stimulated intramolecular signalling within DmpR is a current focus of study in our laboratory.

Experimental procedures

Plasmid constructions

Plasmids were constructed using standard recombinant techniques (Sambrook *et al.*, 1989) and were introduced into *E. coli* strains by transformation and *Pseudomonas* strains by electroporation with a Bio-Rad Gene Pulser. Plasmids based on the phage T7 expression plasmid pET3H expressing DmpR-flag (pVI456) and ΔA2-DmpR-flag (pVI457) have been described previously (Shingler and Pavel, 1995). These derivatives have an *Nde*I site overlapping the start ATG codon and an in frame carboxy-terminal fusion of the Flag epitope (DTKDDDDK; Hopp *et al.*, 1988) as a *Bam*HI to *Hin*dIII linker. An equivalent plasmid expressing ΔD-DmpR-flag (spanning residues 1–496 of DmpR; pVI497) was generated by polymerase chain reaction (PCR) amplification as a *Stu*I–*Bam*HI fragment, with the *Bam*HI site in frame with the flag epitope upon placement between these sites of pVI456. As a specificity control, a plasmid expressing a constitutively active derivative of *Klebsiella pneumoniae* NifA (Perez-Martin and de Lorenzo, 1995a) was generated. ΔA-NifA-flag (amino acids 188–524 with an amino-terminal Met) was constructed by PCR amplification as an *Nde*I to *Bam*HI fragment from plasmid pMC71A (Buchanan-Wollaston *et al.*, 1981), with replacement of the fragment between these sites of pVI456 to generate pVI498. A-domain-flag derivatives expressing different portions of DmpR were generated in an analogous way, resulting in pVI500 (A-1-245-flag), pVI501 (A-1-235-flag), pVI502 (A-1-215-flag), pVI503 (A-1-210-flag), pVI504 (A-1-205-flag) and pVI505 (A-1-200-flag). A-1-235-His6 (pVI510) and A1-210-His6 (pVI511) were generated by replacing the *Bam*HI–*Hin*dIII flag tag linker of the appropriate plasmid with a linker encoding six consecutive His residues followed by a termination codon.

For *in vivo* co-expression studies, A-1-235-flag was placed under the control of the P*tac* promoter on pVI521. This was achieved by cloning the *Nde*I to *Hin*dIII fragment from pVI501 between these sites of pVI520, a broad-host-range RSF1010 $lacI^Q$/P*tac* expression vector based on pMMB66EH (Fürste *et al.*, 1987) that had been modified to contain a polylinker. *Nde*I – *Sma*I – *Bam*HI – *Not*I – *Pst*I – *Hin*dIII downstream of the promoter. For co-expression of A-1-235-flag and DmpR derivatives in intact *Pseudomonas* cells, a RSF1010-compatible RK2-based vector, pVI530, was constructed from pRK2501-E (Lodge *et al.*, 1990) by insertion of a polylinker (*Kpn*I – *Ssp*I – *Hpa*I – *Bam*HI – *Hin*dIII) into the unique *Hin*dIII site within the kanamycin resistance gene. The *dmpR* gene expressed from its native promoter was cloned as a blunt-ended *Not*I fragment from pVI401 (Shingler and Moore, 1994) into the *Hpa*I site of pVI530 to generate pVI531. To obtain expression of the C-domain of DmpR, plasmid pVI532, with an initiation Met codon fused to codons for residues L-219 to G-496 of DmpR, was constructed sequentially using the pVI530 RK2-based vector. This plasmid contains a PCR-generated *Bgl*II to *Nde*I P*trp* promoter and ribosome binding site fragment fused to an *Nde*I to *Pst*I fragment encoding the amino-terminal of ΔA2-DmpR, which in turn was fused to a PCR-generated *Pst*I to *Bam*HI fragment encoding the carboxy-terminal part of the C-domain. Finally, a *Bam*HI to *Hin*dIII linker was used to introduce termination codons in all three reading frames immediately downstream of codon G496. Fidelity of the PCR-generated fragments was confirmed by DNA sequencing.

Affinity enrichment of His epitope-tagged DmpR derivatives

The plasmids expressing A-domain-His6 derivatives from the T7 promoter were introduced into *E. coli* BL21(DE3) plysS (Rosenberg *et al.*, 1987). Fresh transformants were resuspended in Luria broth (LB; Sambrook *et al.*, 1989) containing appropriate antibiotics and grown at 18°C (to facilitate solubility of the protein) until the cultures had reached $A_{650} = 1.2–1.4$. For each preparation, approximately 15 g wet weight of cells

custom synthesized by Amersham. Binding assays were performed with 10 μl of affinity-purified flag-tagged protein bound to beads in a total 200 μl assay buffer prepared as described above. Assays contained the indicated concentration of [^{14}C]-phenol and, where appropriate, 500 μM cold phenol. Under these conditions at 30°C, equilibrium binding was achieved in < 10 min. Samples were incubated with shaking for 25 min at 30°C before loading on a 300 μl 45% sucrose cushion. Protein-bound phenol was separated from free phenol by centrifugation through the cushion with a bench microfuge at 14 000 r.p.m. The radioactivity in the upper layer containing the free phenol and the lower layer containing the protein-bound phenol was recovered independently and analysed by scintillation counting. For the [^{14}C]-phenol binding curve of DmpR-flag, preparations contained 8 μg of protein beads (124 pmol) 10 μl^{-1}. Specific binding of phenol was calculated by the subtraction of non-specific binding observed in the presence of 500 μM competing cold phenol. For comparison of the binding abilities of different flag-tagged derivatives in the presence of 16 μM [^{14}C]-phenol, specific [^{14}C]-phenol binding is expressed as a percentage of that achieved by DmpR-flag, with ΔA-NifA-flag used as a negative control.

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